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Previous studies demonstrated the protective effect of our novel iodine formulation against SM-induced skin lesions. In order to improve the preparation we investigated the inflammatory processes occurring upon SM exposure and particularly those involve cyclooxygenase activity. To evaluate the role of cyclooxygenase-1 and –2 (COX-1, COX-2) in sulfur mustard-induced skin toxicity, we applied the agent to the ears of wildtype (WT) and COX-1- and COX-2-deficient mice. In the latter, ear swelling 24 and 48 h after exposure was significantly reduced by 55% and 30%, respectively, compared to WT These findings were confirmed by histopathological evaluation. The COX-2 inhibitor celecoxib resulted in significant reductions of 27% and 28% in ear swelling at intervals of 40 and 60 min between exposure and treatment, respectively. These findings encouraged us to introduce celecoxib, together with the steroidal anti-inflammatory agent clobetasol, into our iodine preparation. After optimization procedures, the ointment was efficacious 60 min (or less) following exposure. The ability of low iodine concentrations to scavenge reactive oxygen species and to inhibit oxidative burst of activated mouse neutropils might explain the protective mechanism of action of iodine. Previous results have shown that prophylactic injection of H2A histone fragment protected against SM-induced skin lesions. In the present report we found that the T1/2 of the peptide in mouse and humans serum was 7 and 14 min. We also identified the cleavage sites of the peptide by mouse and human serum proteinases; and accordingly synthesized N-methylated analogs for stabilizing the peptide against this proteolytic activity. Injection of the peptide 30 min after exposure significantly reduced ear swelling in the mouse edema model. The structural requirements of the peptide showed that addition of a basic residue to the amino terminal or omission of 2 amino acids from the carboxy terminal produced peptides that were active by post exposure treatment.

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INTRODUCTION

Sulfur mustard (SM) is a potent vesicant employed as a chemical weapon in various conflicts during the 20th century (1, 2). It functions as a powerful alkylator and highly cytotoxic blisterogen in both humans and animals (1-9). Skin exposed to SM develops erythema within 30 minutes to several hours after exposure followed by edema, vesicle and blister formation, ulceration, necrosis and desquamation (2, 7, 8, 10). The severity of lesions observed after exposure to SM has emphasized the need for an efficient pharmacological antidote against its vesicating activity. The powerful alkylating activity of SM (11-13) results from its conversion, in aqueous solution, to the highly electrophilic ethylene episulfonium derivative (2) which can be neutralized by nucleophilic agents. Protection against SM might be achieved by glutathione derivatives (14, 15), cysteine esters (16) and a cysteine precursor (17). Despite having some beneficial effects, these agents were not efficacious enough to be used as antidotes. Additional agents, such as arginine analogs (18-22), a calcium channel blocker (23), niacinamide (24) and its combination with promethazine and indomethacin (25) exhibited weak therapeutic effects as post-exposure treatment against SM in experimental animals, although some of these agents were beneficial in *in vitro* and *in vivo* systems, particularly if treated prophylactically. Therefore, we undertook the development of efficacious topical antidote for treatment of mustard gas-exposed victims.

BODY

IODINE PROTECTS AGAINST SM

In an attempt to adopt a different approach for addressing this issue, we assumed that the divalent sulfur atom of SM [$S(CH_2CH_2Cl)_2$] can be oxidized to form its sulfoxide form [$SO(CH_2CH_2Cl)_2$], an inactive derivative of the vesicant (26). The most suitable oxidizing agent was iodine, a widely used topical antiseptic agent. We demonstrated that topical application of povidone-iodine 5 and 10 min after skin exposure to SM significantly reduced tissue damage as compared to the control sites treated with SM only (27-33). A lower degree of protection was observed at a 20 min interval between exposure and treatment. However, practically, 10 min is too short an interval for treatment of the soldier in the battlefield or of a civilian in case of gas attack. At that stage support by the USAMRMC began (Cooperative Agreement No. DAMD17-98-2-8009). Our main objective was ***to extend the time interval between exposure and treatment*** by improving the iodine formulation. Our experience showed that iodine is more efficacious than povidone-iodine, a polyvinylpyrrolidone-iodine complex (27-29, 34). The currently available iodine preparations contain sodium or potassium iodide, believed to form the water-soluble I_3^- ion. However, the efficacy of the new iodine formulation was significantly improved due to employment of tetraglycol as solvent without iodide addition; (a patent (34) was filed) which enables 50% water content without iodine precipitation. It is assumed that under these circumstances iodine retains its molecular form i.e. I_2, a more hydrophobic molecule than I_3^-, resulting in enhanced skin penetration and improved biological activity.

Indeed, ***post-exposure treatment with this new iodine preparation resulted in a significant reduction in skin ulceration area at time intervals between exposure and treatment of 30 min and less, and to a lesser extent at 45 min***. These findings were corroborated by histopathological examination of SM-exposed skin treated with iodine 30 min after intoxication; statistically significant reductions of 35, 67, 43, 39, and 45% were observed in subepidermal microblister formation, epidermal ulceration, dermal acute inflammation, hemorrhage and necrosis, respectively. These findings were recently published (34-41) and, together with being non-toxic and safe preparation, encouraged us to further improve the iodine preparation in order to extend the interval between exposure and treatment.

THE INVOLVEMENT OF INFLAMMATORY MEDIATORS AND CYCLOOXYGENASE (COX) SYSTEM IN SM-INDUCED SKIN TOXICITY

The evolution of SM-induced skin lesion involves a variety of inflammatory processes and production of proinflammatory factors (42-48). The dermal infiltration of polymorphnuclear cells upon SM exposure (35, 47) further supports the involvement of inflammation in skin irritation induced by this vesicant. These findings stimulated the introduction of non-steroidal antiinflammatory drugs (NSAIDs) into preparations against SM-induced skin lesions. Buxton et al. (49), Babin et al. (50) and Zhang et al. (51) have all shown the protective activity of topical and parenteral treatments with NSAIDs, including indomethacin and olvanyl, against skin toxicity of SM.

Despite the information about COX involvement in SM-indcued dermal toxicity, no information is available on the role of COX isoforms, namely, COX1 and COX2 in

skin toxicity caused by SM. In order to address this issue we adopted the following approaches:

a) Testing toxicity of SM in COX-1- and COX-2-deficient mice (preliminary results were described in the final report of Cooperative Agreement No. DAMD17-98-2-8009).

b) Immunohistochemical staining of COX-2 isozyme in normal mice exposed to SM.

b) Testing the effect of COX-2 selective inhibitors against SM-induced skin toxicity in normal mice.

c) Testing iodine preparation containing COX-2 selective inhibitor.

EXPERIMENTAL PROCEDURES

Mouse ear swelling test. Male COX-1- and COX-2-deficient mice and their WT were anesthetized by sodium pentobarbital, 60mg/kg ip [0.1ml/25g body weight (BW) of 1.5% solution], and placed on their abdomens. Anesthesia was maintained by using 0.03ml/25g BW whenever needed. The outer side of the ear was exposed to 0.317 mg SM (5µl of 1.27 mg SM diluted in 20 µl dichloromethane). Dosage was not dependent on BW. Mouse ear thickness was measured at study start and 24 and 48 h after exposure using a micrometer (Model PK-0505, Mitutoyo Corporation, Japan). Edema was assessed by the difference between ear thicknesses measured prior to and

after exposure. Animals were sacrificed 48 h after exposure using 100 mg/kg sodium pentobarbital ip. Ear specimens were removed, fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned at 5-6 μm, and stained with hematoxylin and eosin (H&E) for histopathological evaluation.

In a separate experiment, ears of male ICR mice were exposed to 0.317 mg SM. Ears of the exposed and control groups were removed 8 h after treatment, fixed in formalin for 24 h, then kept in 70% ethanol for COX-2 immunohistochemical staining.

In an additional series of experiments normal male ICR mice were ip-injected with 20 mg/kg celecoxib (dissolved in 1,2 propanediol) 20, 40, and 60 min after exposure to SM. Control animals received ip injection of the vehicle. Measurement of parameters was conducted as described above.

Immunohistochemical evaluation of COX-2. Paraffin-embedded ear samples after SM exposure and controls were stained as described by Nyska et al. (2001). Immunohistochemistry was performed using the avidin-biotin-peroxidase method. Sections were stained with anti COX-2 antibody (Caymen Chemical, Ann Arbor, MI). Following deparaffinization through xylene and a series of graded alcohols, all slides were placed in 1X Automation Buffer (AB) (Biomeda, Foster City, CA) and blocked for endogenous peroxidase activity with 3% H_2O_2 for 15 min.Heat-induced epitope retrieval was completed for all slides by incubating the slides in 1X citrate buffer, pH 6.0 (Biocare Medical, Walnut Creek, CA). Slides were heated for 5 min in a microwave oven at 50% power for 2 cycles. Between cycles,

fresh citrate buffer (50 ml) was added to the Tissue-Tek (Sakura, Torrance, CA) container. After microwaving, the slides were allowed to cool for 15 min, then rinsed in distilled water, and placed in 1X AB. All incubations were carried out in a humidified chamber at room temperature. Slides were blocked in 5% normal goat serum (Jackson Immunoresearch, West Grove, PA) and then incubated with the primary antibody at a dilution of 1:300 for 1 h. Normal rabbit serum (Jackson Immunoresearch) was used as the negative control. The positive control was a super-ovulating mouse ovary; the animal was subcutaneously injected with 5 IU of pregnant mare serum gonadotrophins (PMSG), followed in 48 h later by 5 IU of human chorionic gonadotrophin (HCG), and killed 8 h later. Slides were washed in buffer; incubated with the secondary antibody, biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) for 30 min; washed again; and then labeled with an avidin-biotin complex (Vector Elite Kit, Vector) for 30 min. Slides were rinsed in water, counterstained with Harris hematoxylin (Harelco, Gibbstown, NJ) and coverslipped with Permount (Surgipath, Richmond, IL)

Histopathological evaluation. Each mouse ear section was evaluated in a blinded manner by the investigator, Dr. Abraham Nyska, without his knowing the identity of the treatment group and scored for histopathological changes. In the mouse ear the entire section, including inner and outer sides, was evaluated. The reactive and inflammatory changes in the epidermis and dermis were assigned severity grades of 0-4 representing unremarkable, minimal, mild, moderate, and marked changes, respectively. Epidermal parameters included subepidermal microblister formation,

ulceration, necrosis, and crust formation. Dermal parameters included hemorrhage, acute inflammation, and necrosis.

RESULTS

SM-induced skin toxicity in COX-1- and COX-2-deficient mice

Macroscopic observations

Macroscopic observations showed statistically significant reductions in COX-2-deficient mice of 55% and 30% in ear swelling 24 and 48 h after exposure, as compared to the WT response (Fig. 1). No differences were observed between COX-1-deficient and the WT mice.

Histopathological evaluation

The gross findings were corroborated by the histopathological evaluation performed 48 h after exposure when the histological changes were prominent and assessable. A photographic presentation of characteristic findings is shown in Fig. 2 showing intact epidermis in COX-2-deficient mice; epidermal ulceration and epidermal necrosis in COX-1-deficient mice; and, in WT mice, subepidermal microblister formation and apoptotic and coagulative necrosis of the epidermis. No epidermal ulceration was seen in COX-2-deficient mice, whereas the WT mice exhibited some degree of this parameter (Fig. 2). Quantification of these parameters (Fig. 3) showed significant reduction in severity of subepidermal microblister formation (44%) epidermal necrosis (29%), acute inflammation (42%), hemorrhage

(25%) and dermal necrosis (30%) in COX-2-deficient mice, as compared to the WT mice. Nevertheless, COX-1 deficiency resulted in an exacerbation in severity of epidermal ulceration (4.6 fold), while a similar trend was observed with epidermal necrosis. A comparison between COX-1- and COX-2-deficient mice revealed statistically significant differences in all measured histological parameters (Fig. 3).

COX-2 immunohistochemistry

In order to investigate the very early changes in COX-2 expression upon SM exposure, we performed COX-2 immunohistopathology of normal mice 8 h after SM exposure (Fig. 2H) and control mice (Fig. 2G). Sulfur mustard induced swelling of the cytoplasm of the fibroblasts and mononuclear cells in the dermis with dispersion of the COX-2 cytoplasmic positive granules from the fibroblasts and other mixed inflammatory cells. In contrast, fibroblasts and mononuclear cells present in the dermis of control mice had relatively scant, dense, homogenous granular COX-2 stained cytoplasm. The change in the cytoplasmic aspect of COX-2 staining suggests activation and discharge of the preexisting granules.

Effect of selective COX-2 inhibitor on SM-induced skin toxicity

Macroscopic observation

By macroscopic observation, a single post-exposure treatment of normal male ICR mice with the selective COX-2 inhibitor celecoxib resulted in significant reductions of 27% and 28% in ear swelling at intervals of 40 and 60 min between exposure and treatment, respectively (Fig. 4).

Histopathological evaluation

Histopathological evaluation showed significant reductions in subepidermal microblister formation (73%) and dermal necrosis (32%), compared to the control group (Fig. 5). The nonselective COX inhibitor ibuprofen exerted a weaker effect against SM-induced ear toxicity (data not shown).

Effect of topical treatment with iodine preparation containing celecoxib

Effect of different polymer compositions

The ability of celecoxib to reduce SM-induced skin toxicity led us to incorporate this COX-2 selective inhibitor into the iodine formulation together with the steroidal anti-inflammatory agent clobetasol. We have tested several compositions of the polymers polyvinylpyrrolidone (PVP) and polyvinylalcohol and realized that composition 1 was the most efficacious formulation (Fig. 6).

We tested various kinds of polymers, combination of polymers and micro-emulsion systems, however, none of them showed the efficacy of that composed of shown in Fig. 6.

Effect of different iodine concentrations in formulation containing clobetasol and celecoxib

As shown in Fig. 7, the maximal protective effect was obtained with preparation containing 2% iodine (together with celecoxib and clobetasol).

Effect of the iodine formulation at various intervals between exposure and treatment

Fig. 8 demonstrates the efficacy of the preparation applied 60 min after exposure. Treatment at shorter intervals between exposure and treatment was efficacious as well.

Multiple treatments

The described findings are based on the protocol of a single topical treatment after exposure. In an attempt to further improve the efficacy of iodine preparation the animals were daily treated with the ointment. The multiple treatments procedure did not significantly improve its protective effect. The reason for that might stem from the fact that the most crucial inflammatory processes occur during the first hours after exposure—at that time the iodine exerts its protective effect; later on, in range of hours and days after exposure, the preparation is helpful anymore.

Mechanism of action of the protective effect of iodine

Effect of iodine on oxidative burst of activated mouse neutrophils

In attempt to verify the mechanism of the protective action of iodine, we tested the capability of iodine to prevent oxidative burst from mouse neutrophils stimulated by phorbol myristate acetate (PMA). The reaction mixture contained neutrophils (collected from peritoneal cavity of mouse ip injected with thioglycolate 24 hours prior the experiment), Hank's balanced salt solution containing glucose, luminol, iodine at the indicated concentrations (except of the control) and 2.5μM PMA. The

peak value of chemiluminescence (after 3 min of reaction) is shown in Fig. 9 for each iodine concentration.

The concentration-dependent inhibition of oxidative burst is shown in Fig. 9. A pronounced effect can be observed in the range of 0.0025%-0.005% iodine.

In an attempt to verify the mechanism of the inhibition, we tested the effect of iodine on a cell-free radical generating system containing glucose oxidase that catalyzes the formation of hydroxyl radical and gluconic acid from glucose and oxygen. As with neutrophils, the same range of iodine concentrations inhibited the chemiluminescence-induced glucose oxidase (Fig. 10), indicating that iodine is operating by scavenging the reactive oxygen radicals produced during the SM-induced inflammatory processes.

Protective effect of H2A histone fragment agasint SM-induced skin lesions

As previously described, we isolated and identified H2A fragment of 9 amino acids from the guinea pig with the following sequence:

H-Lys-Gly-Asn-Tyr-Ala-Glu-Arg-Ileu-Ala-OH (abbreviation: KGNYAERIA)

We have shown that this peptide had protective effect by intreadermal injection in the guinea pig model. Later on we synthesized the mouse/human sequence:

H-Lys-Gly-His-Tyr-Ala-Glu-Arg-Val-Gly-OH (IIIm1) (abbreviation: KGHYAERVG). We reported on the counter-irritating activity of this peptide against SM-induced mouse ear edema by iv administration. There was a reduction of 25% in mouse ear swelling as compared to the control group.

It was assumed that serum proteinases are responsible for rapid degradation of the peptide, hence, its efficacy might be increased by stabilizing its peptide bonds against proteolytic activity of the serum. In order to address this issue the first step was to identify the cleavage sites of the peptide by serum proteolytic activity.

The experimental procedure

The peptide was incubated with mouse serum at 37°C, then the high molecular weight proteins were precipitated with perchloric acid and centrifugation, then the resulting supernatant was injected into HPLC using reverse phase C18 column. The separated peak were collected and identified by mass spectra analysis.

Results

Degradation of IIIM1 and identification of degradation products

HPLC analysis revealed that the T1/2 of IIIM1 peptide in mouse and human serum was 7 and 14 min, respectively.

Fig 11 shows the peptide and its degradation products i.e. KGHYAERVG, GHYAERVG, HYAERVG, YAERVG by mouse serum. It is shown that the serum causes sequential hydrolysis of the amino terminal moieties of the peptide as shown in the following scheme:

H-Lys-Gly-His-Tyr-Ala-Glu-Arg-Val-Gly-OH

The same degradation products were produced by incubation of the peptide with human serum.

Synthesis of N-methylated analogs of IIIM1

A series of N-methylated analogs was synthesized of which the most successful one the His and Tyr moieties were substituted by N-methylated analogs (termed P4) as shown in the following scheme:

H-Lys-Gly-***Me***His-***Me***Tyr-Ala-Glu-Arg-Val-Gly-OH (P4)

Protective activity of P4 by post exposure treatment

Fig. 12 shows that post exposure treatment (subcutaneous injection into the ear base) with 10μg P4 reduced SM-induced ear swelling by 25% (measured 4 days after treatment). Higher or lower doses did not show significant effect.

Structure-activity relationship

Since IIIM1 peptide is a H2A histone peptide (36-44 position), it was of interest to test longer or shorter fragments of this peptide (namely, 35-44, 36-45, 35-45 position etc.). The following peptides were synthesized (parentheses indicate their nicknames):

H-Arg-Lys-Gly-His-Tyr-Ala-Glu-Arg-Val-Gly-OH (P AD1)

H-Lys-Gly-His-Tyr-Ala-Glu-Arg-Val-Gly-Ala-OH (P AD2)

H-Arg-Lys-Gly-His-Tyr-Ala-Glu-Arg-Val-Gly-Ala-OH (P AD3)

H-Leu-Arg-Lys-Gly-His-Tyr-Ala-Glu-Arg-Val-Gly-OH (P AD4)

H-Lys-Gly-His-Tyr-Ala-Glu-Arg-Val-Gly-Ala-Gly-OH (P AD5)

H-Leu-Arg--Lys-Gly-His-Tyr-Ala-Glu-Arg-Val-Gly-Ala-Gly-OH (P AD7)

Fig. 13 shows that the P AD1 derivative in which Arg residue was added into the amino terminal site, was the only active peptide derivative able to reduce SM-induced ear swelling (50μg injected into each ear base, measured 5 days after treatment).

Another kind of peptide derivatization is omission of amino acids from the parent peptide. Interestingly, omission of 2 moieties from the carboxy terminal of IIIm1 produced a bioactive peptide with the following sequence:

H-Lys-Gly-His-Tyr-Ala-Glu-Arg-OH (Pfr7)

Fig. 14 shows that treatment with 50μ Pfr7 resulted in statistically significant reduction of 34% in SM-induced ear swelling when injected 60 min after exposure. Longer or shorter intervals had no significant effect.

KEY RESEARCH ACCOMPLISHMENTS

- The iodine preparation was optimized; the polymer composition was optimized; the combination of the active components was optimized.
- Single treatment was proved to most efficacious procedure; multiple treatments did not improve the results.
- The contribution of each of the active components of the preparation was additive.
- The cleavage sites of the peptide by mouse and human protelytic activities were identified.
- N-methylated analogs were synthesized; of which the most active one was a peptide containing di-N-methylation at Tyr His resuidues. This peptide and others were active against SM by post exposure treatment.

REPORTABLE OUTCOMES

1. Wormser, U., Sintov, A., Brodsky, B., Casillas, R.P. and Nyska, A. Protective effect of topical iodine containing anti-inflammatory drugs against sulfur mustard-induced skin lesions. Arch. Toxicol. in press (2003)

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CONCLUSIONS

The iodine preparation was optimized while single treatment within 60 min after exposure was proved to efficacious procedure. The cleavage sites of the peptide by mouse and human protelytic activities were identified and were a results of aminopeptidase cleavage; Accordingly N-methylated analogs were synthesized while the most active one was a peptide containing di-N-methylation at Tyr His resuidues. This peptide was active at 30 min between exposure and treatment. A fragment containing the seven C-terminal amino acids was active at interval of 60 min between exposure and treatment.

So what: The present study improved the iodine formulation—which was shown to be efficacious within 60 min between exposure and treatment. Post exposure parenteral treatment with the peptide derivatives/analogs provided protection by post exposure treatment.

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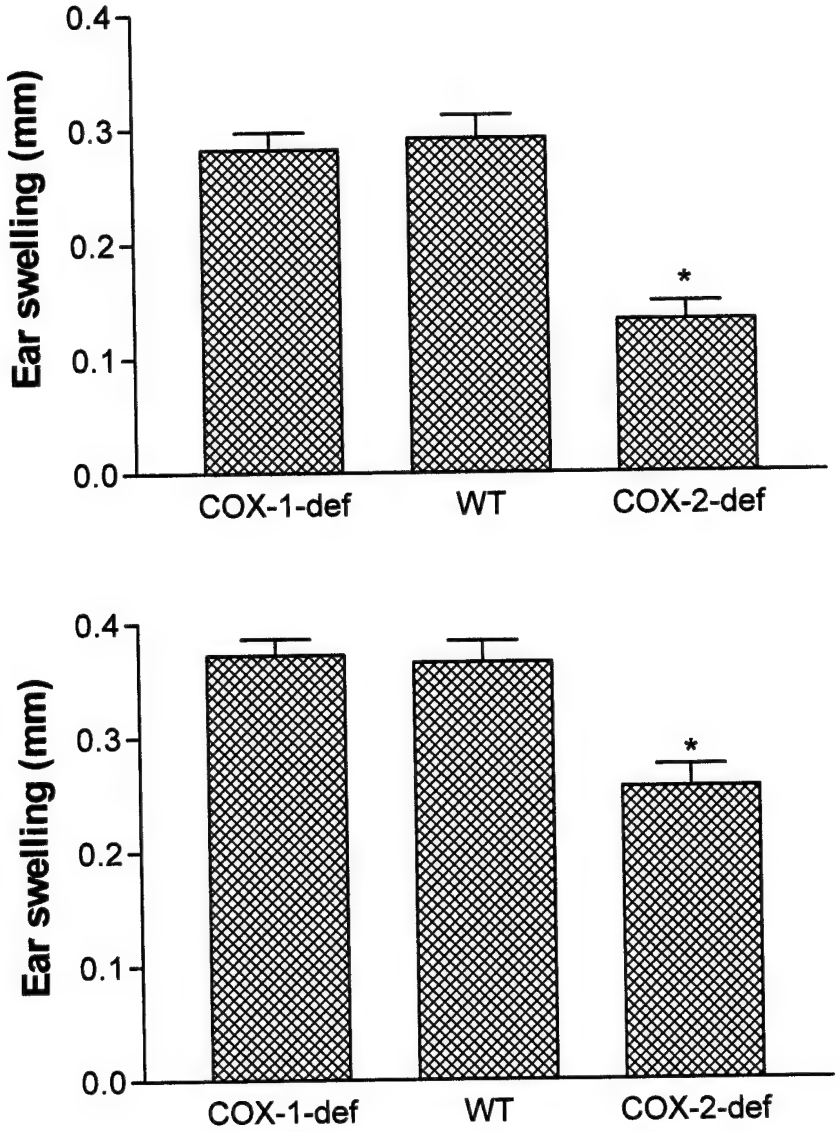
APPENDICES

Fig. 1


Ear swelling (mm)
0.4
0.3
0.2
0.1
0.0
*
COX-1-def
WT
COX-2-def
Ear swelling (mm)
0.4
0.3
0.2
0.1
0.0
*
COX-1-def
WT
COX-2-def

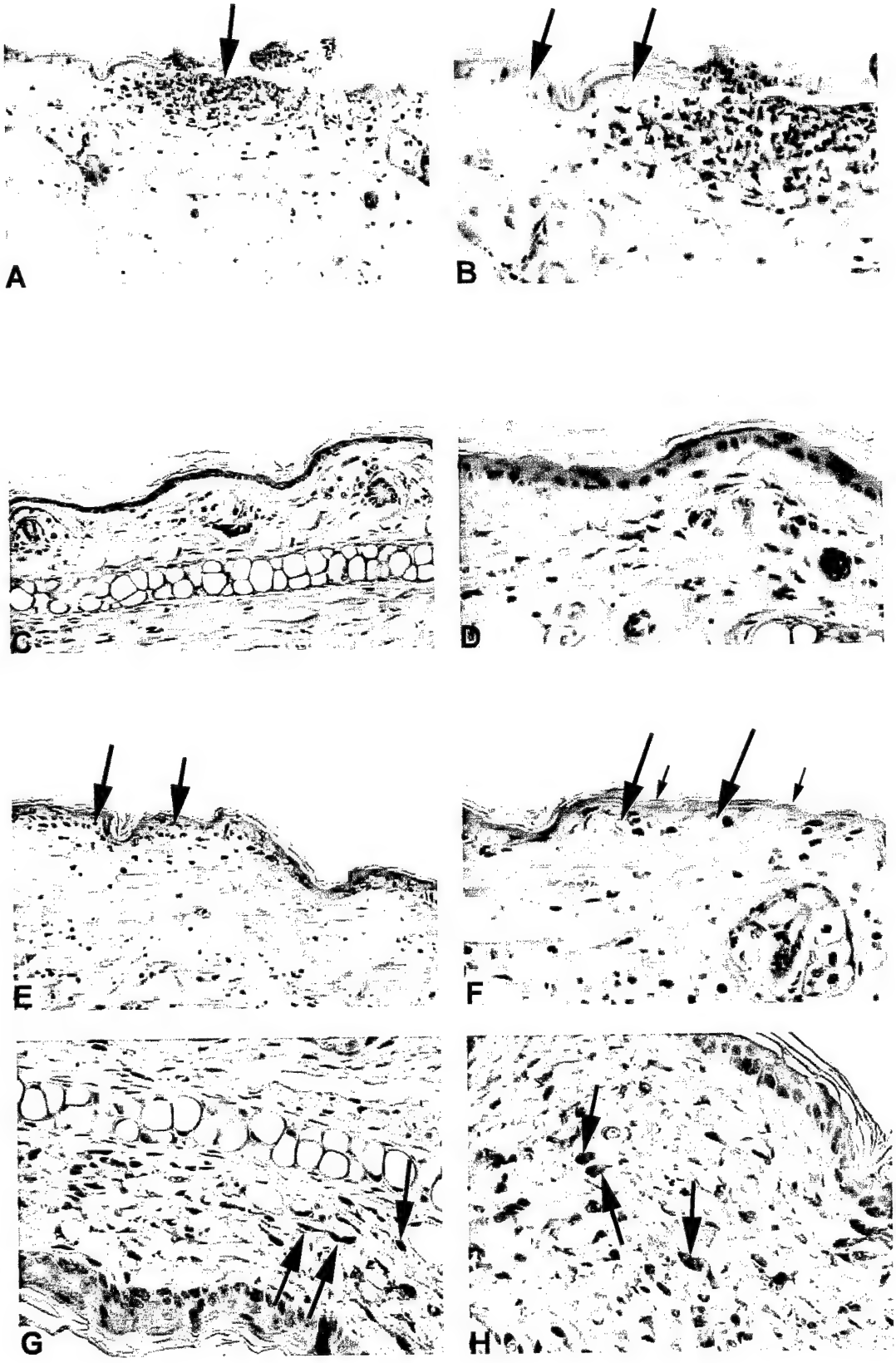


Fig 2

Fig. 3

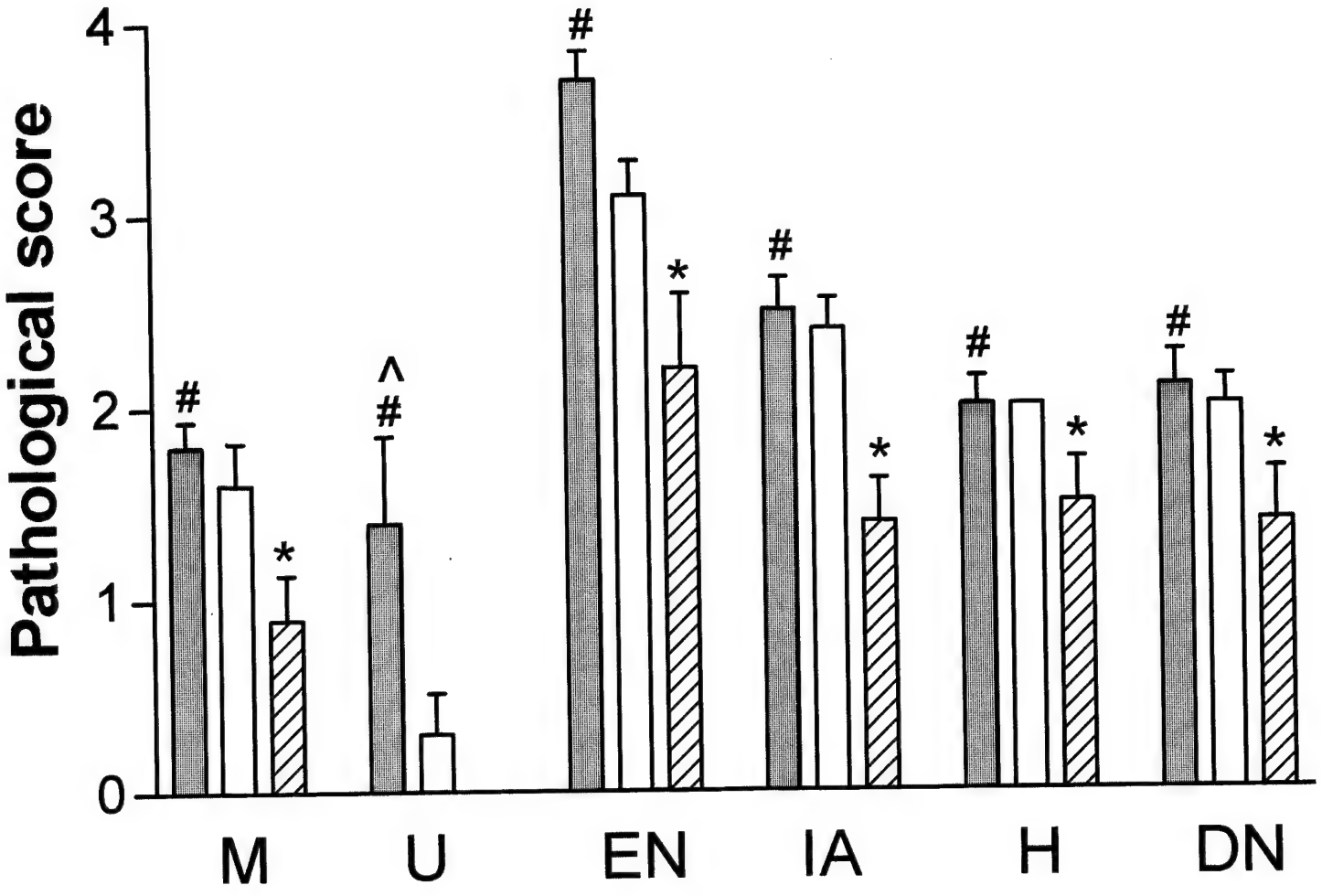


Fig. 4

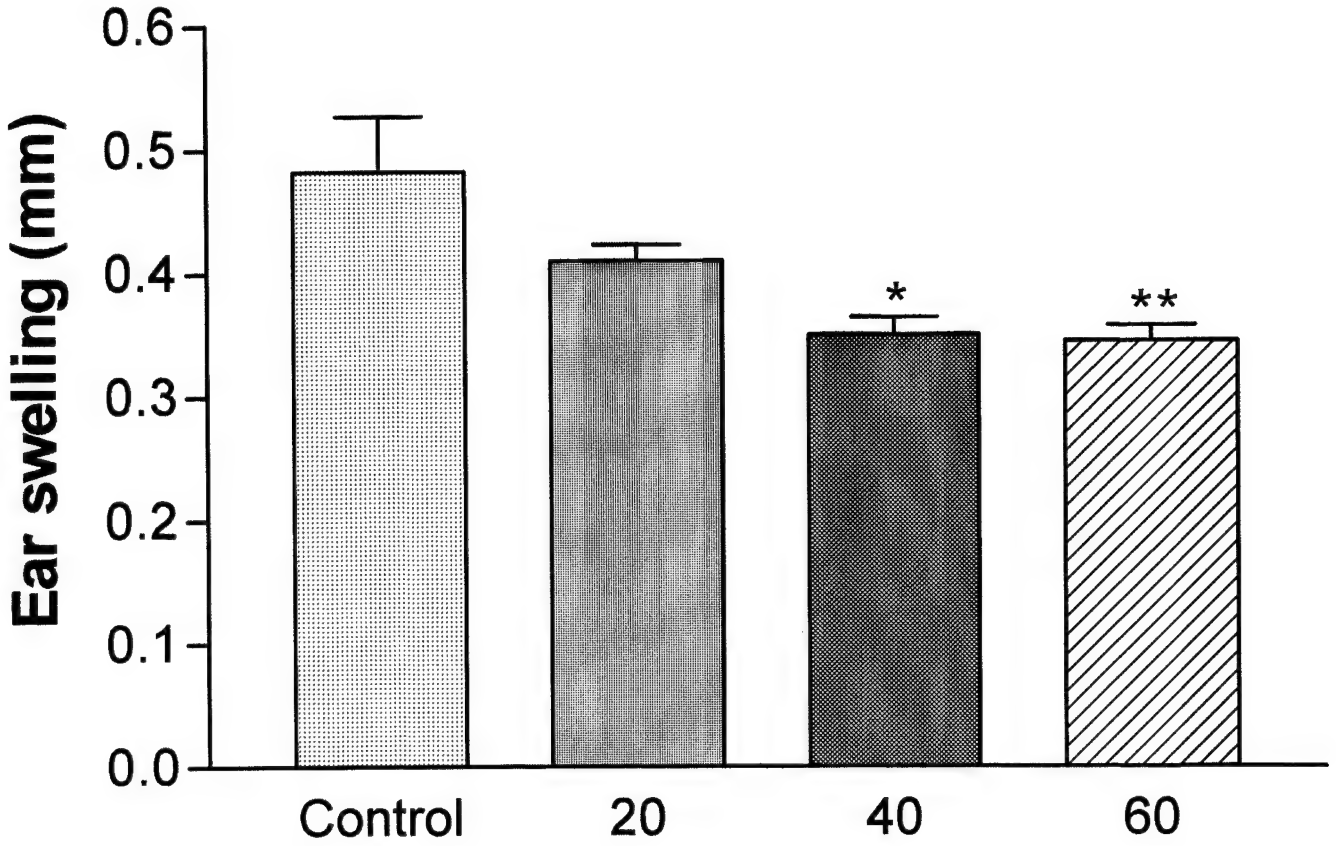


Fig. 5

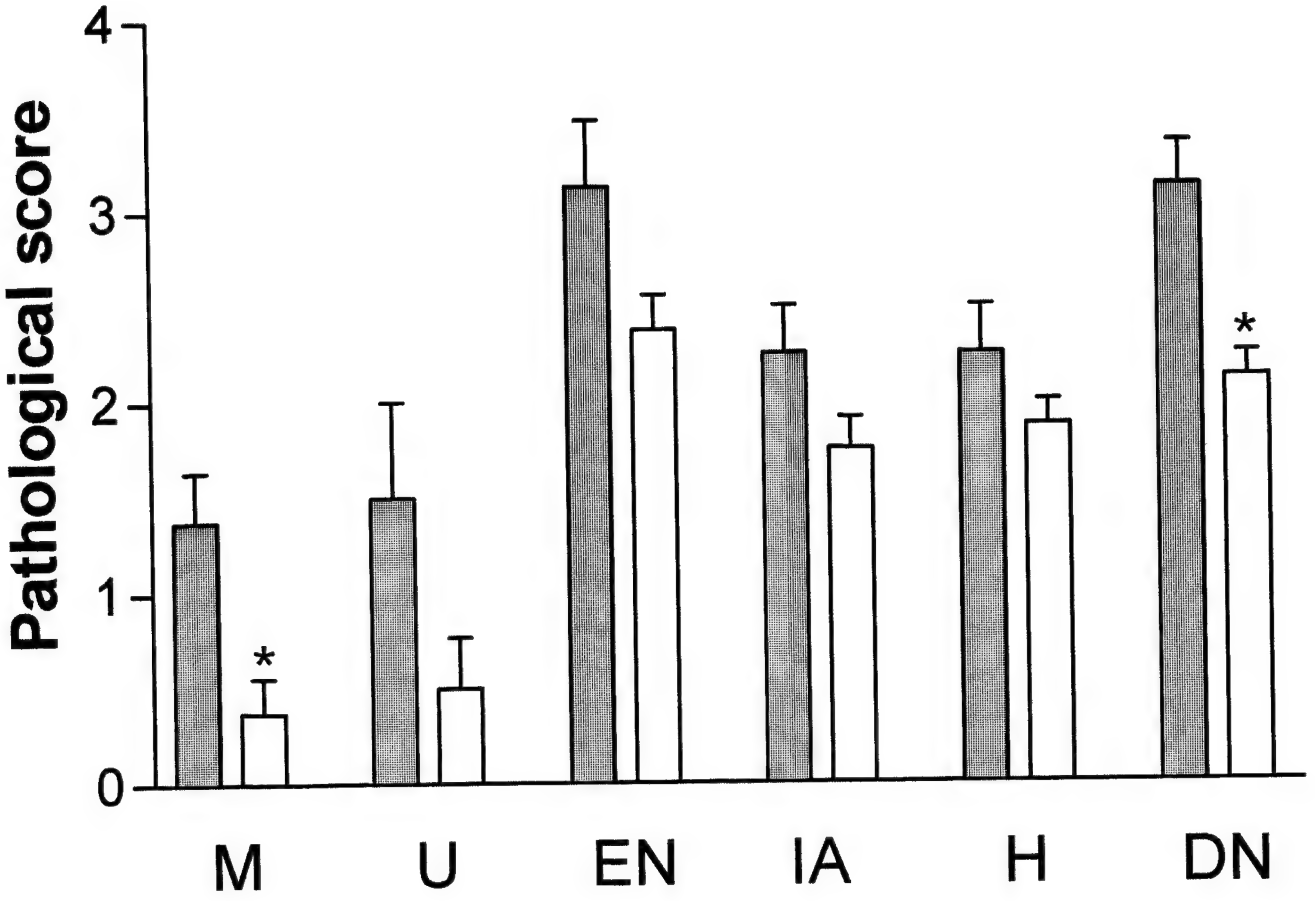
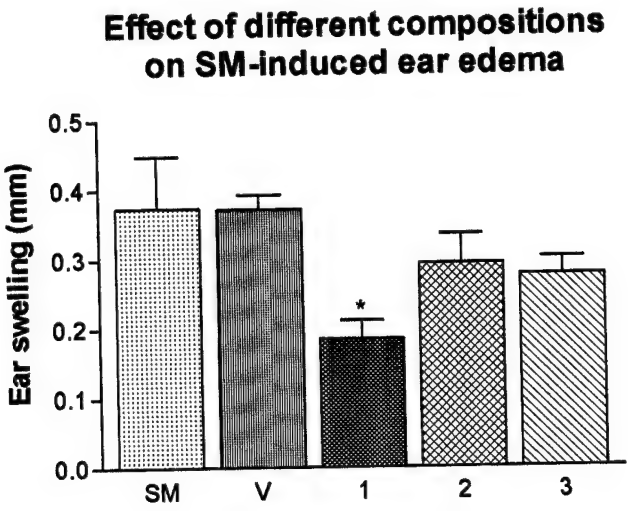


Fig. 6



V - (vehicle) - 30%PVP+2%PVA+50%TG
+DDW (completion to 100%)

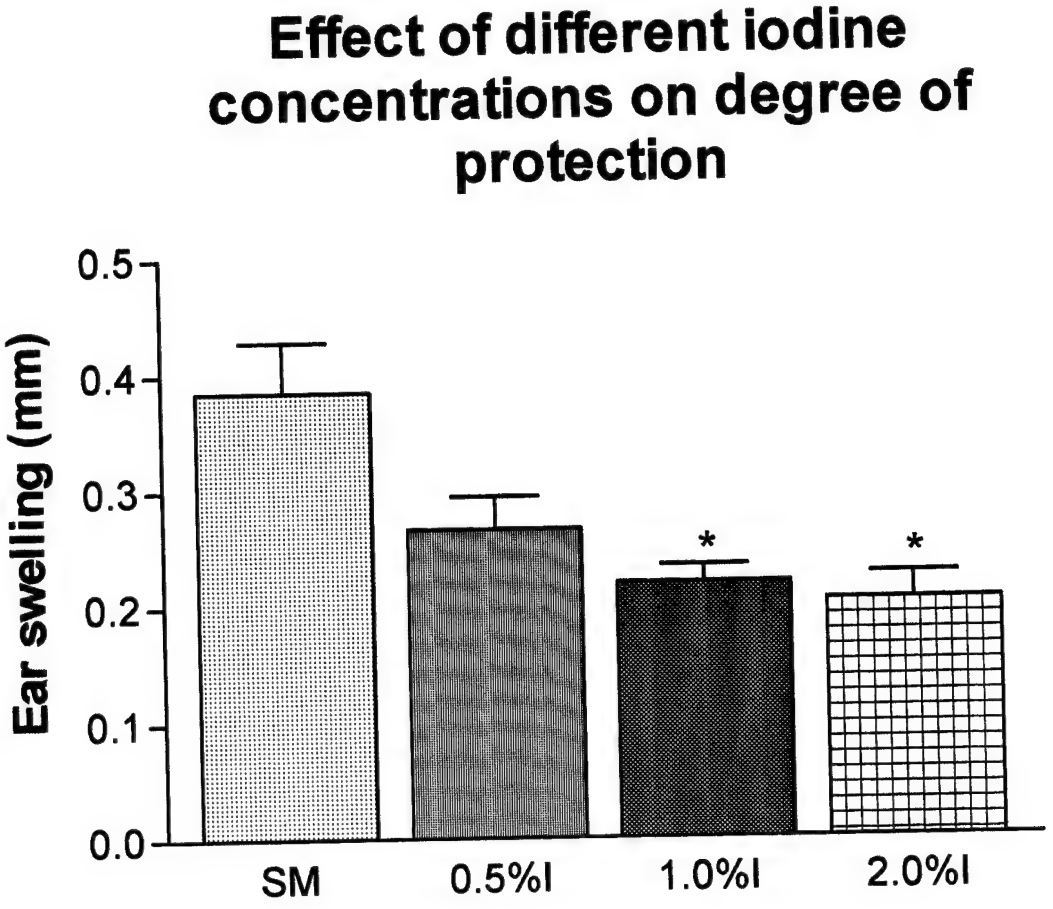
1 - 2%I+0.1%Clobetasol+0.5%Celecoxib+
30%PVP+2%PVA+50%TG+DDW

2 - 2%I+0.1%Clobetasol+0.5%Celecoxib+
29.5%PVP+1.75%PVA+50%TG+DDW

3 - 2%I+0.1%Clobetasol+0.5%Celecoxib+
27,5%PVP+1%PVA+50%TG+DDW

*$p<0.05$

Fig. 7


Effect of different iodine concentrations on degree of protection
Ear swelling (mm)
0.5
0.4
0.3
0.2
0.1
0.0
*
*
SM
0.5%I
1.0%I
2.0%I

*p<0.02

Fig. 8

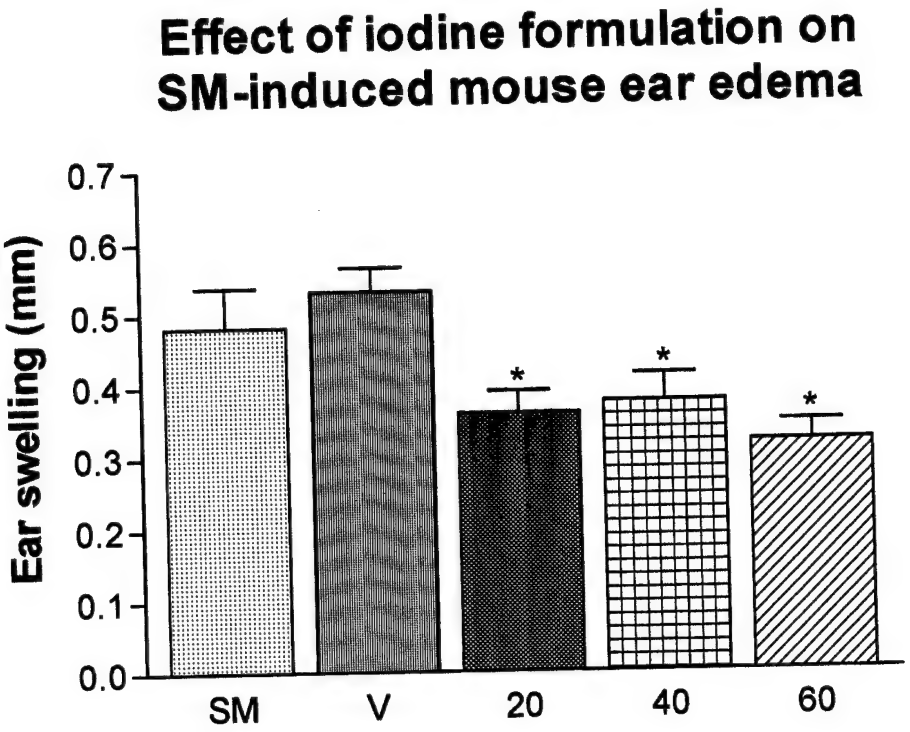

Effect of iodine formulation on SM-induced mouse ear edema
Ear swelling (mm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
*
*
SM
V
20
40
60
Time intrevals between exposure and treatment (min)
V=vehicle applied 40 min after exposure; SM=irritant only; Results were taken 5 days after treatment; p<0.02

Fig. 9

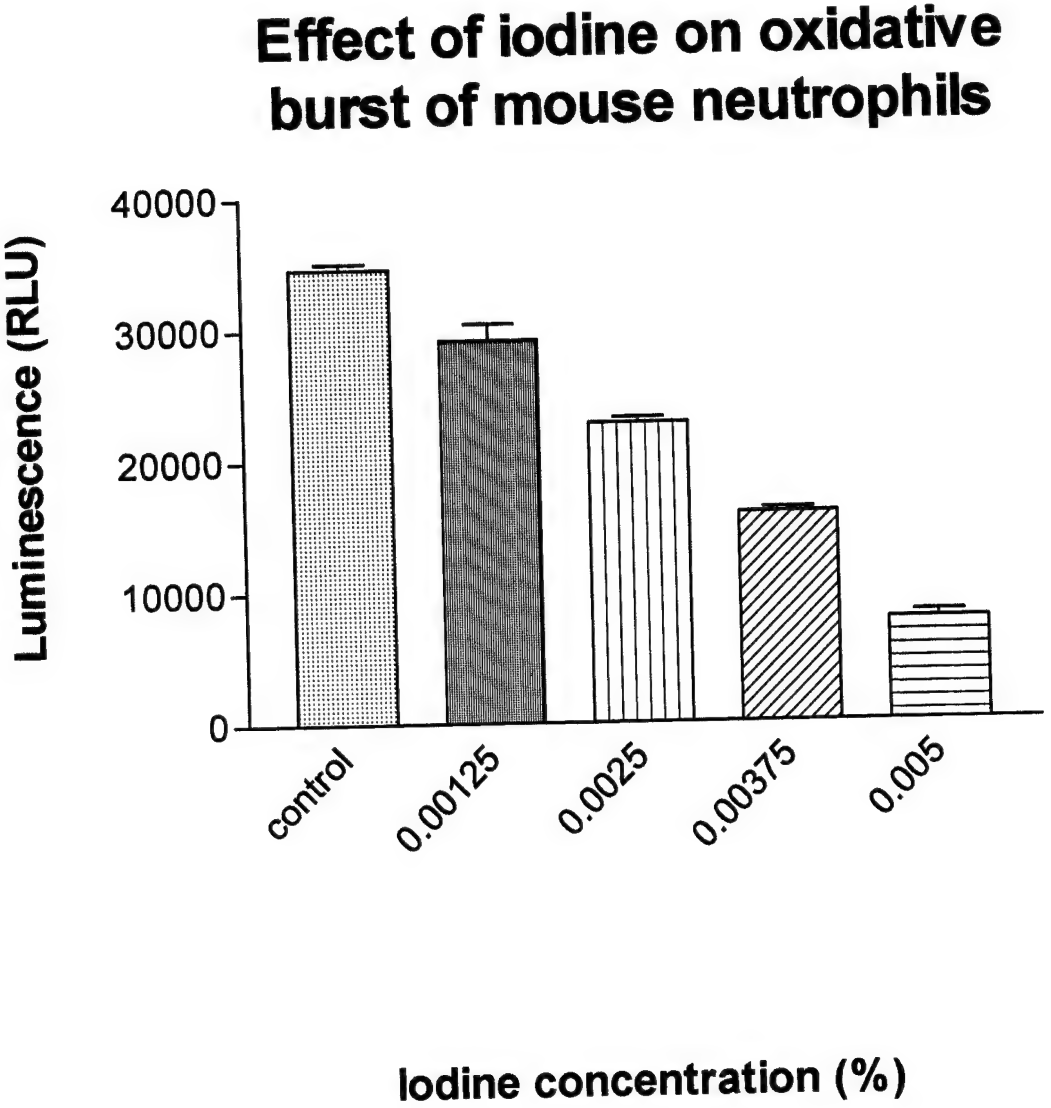

Effect of iodine on oxidative burst of mouse neutrophils
Luminescence (RLU)
40000
30000
20000
10000
0
control
0.00125
0.0025
0.00375
0.005
Iodine concentration (%)

Fig. 10

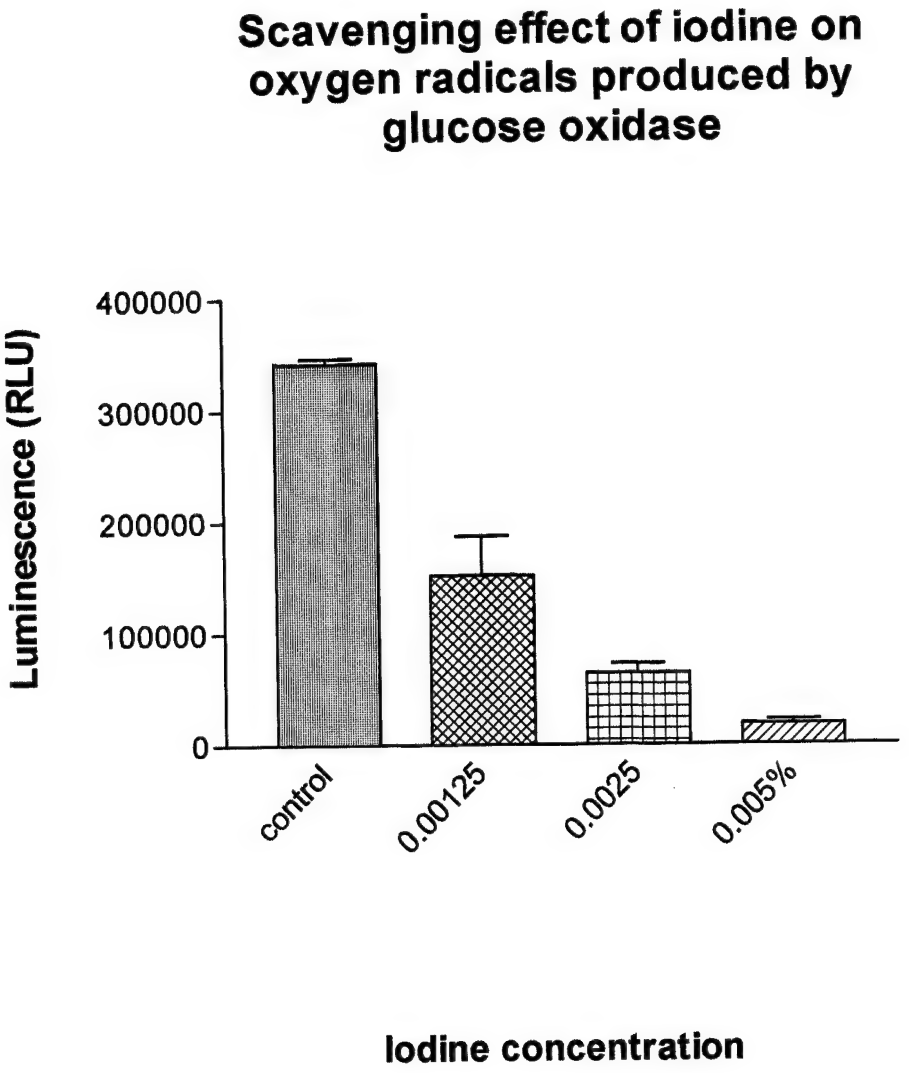
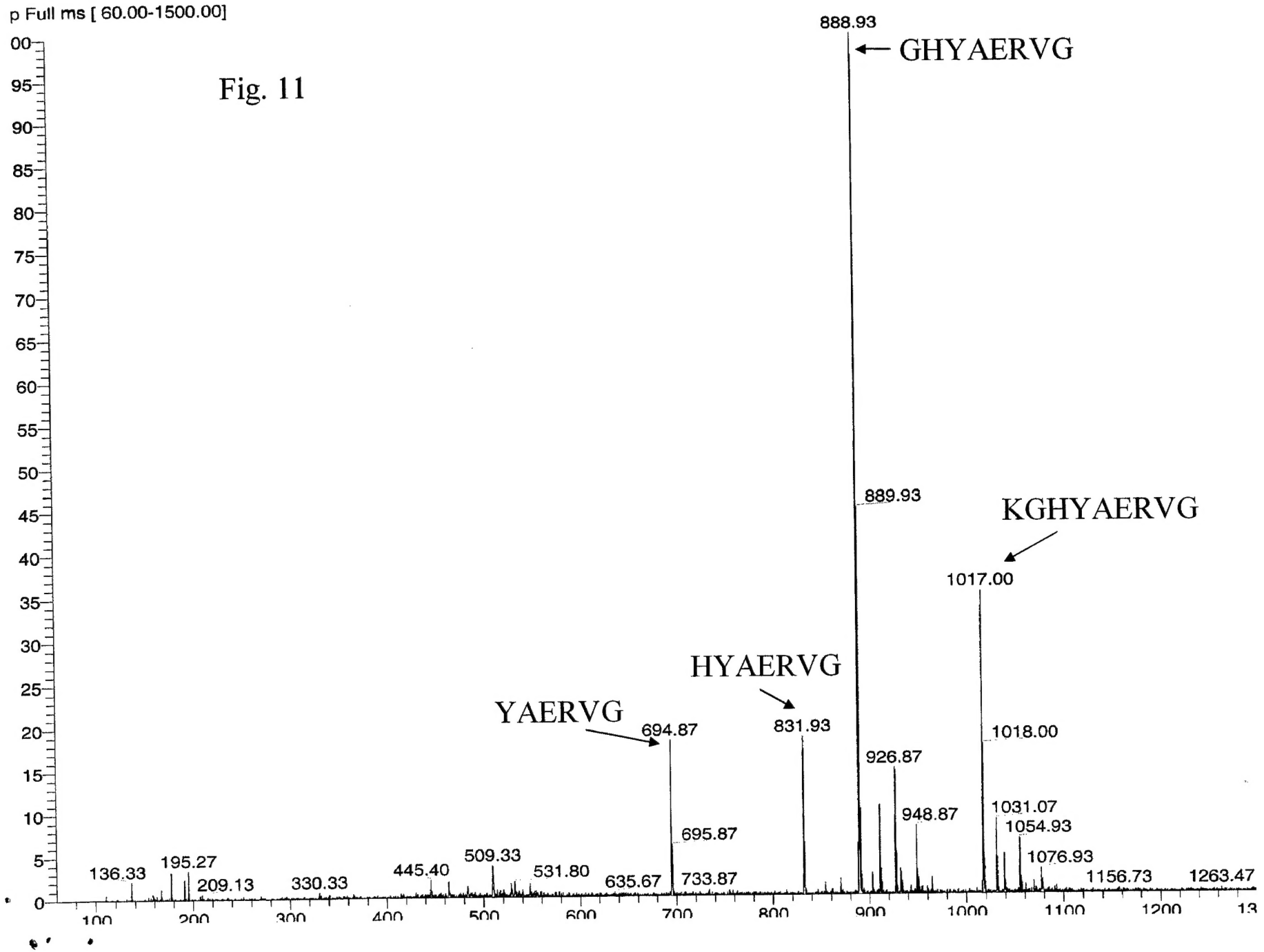

Scavenging effect of iodine on oxygen radicals produced by glucose oxidase
Luminescence (RLU)
400000
300000
200000
100000
0
control
0.00125
0.0025
0.005%
Iodine concentration

Fig. 11


p Full ms [60.00-1500.00]
888.93
GHYAERVG
889.93
KGHYAERVG
1017.00
HYAERVG
831.93
YAERVG
694.87
1018.00
926.87
948.87
1031.07
1054.93
1076.93
695.87
733.87
1156.73
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136.33
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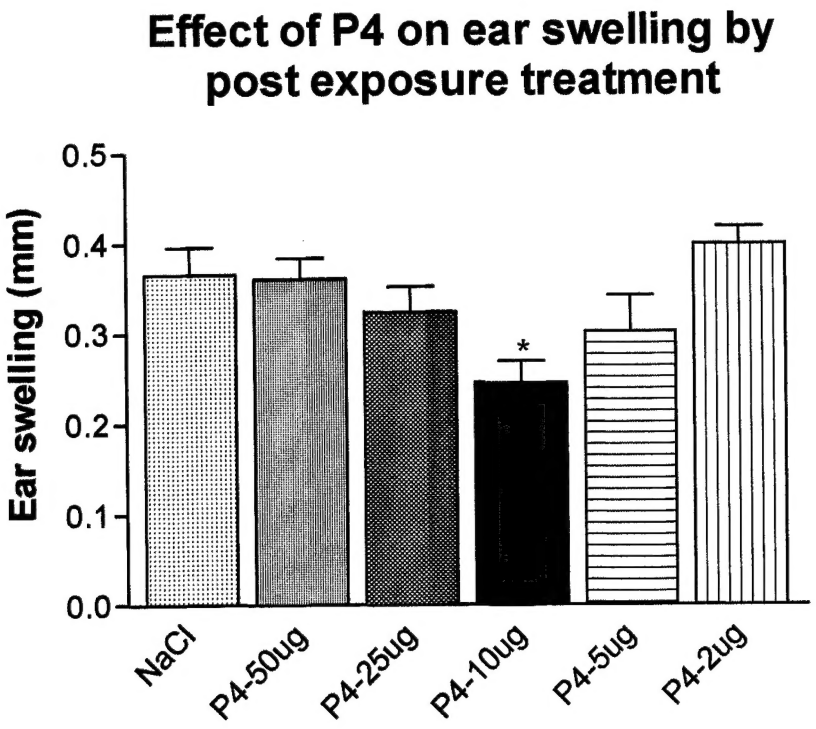
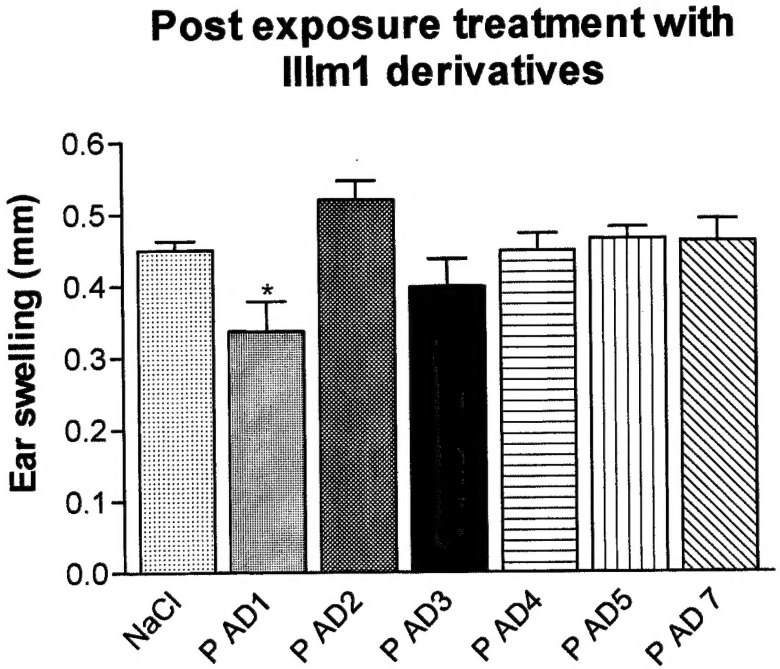
209.13
330.33
445.40
509.33
531.80
635.67

Fig. 12



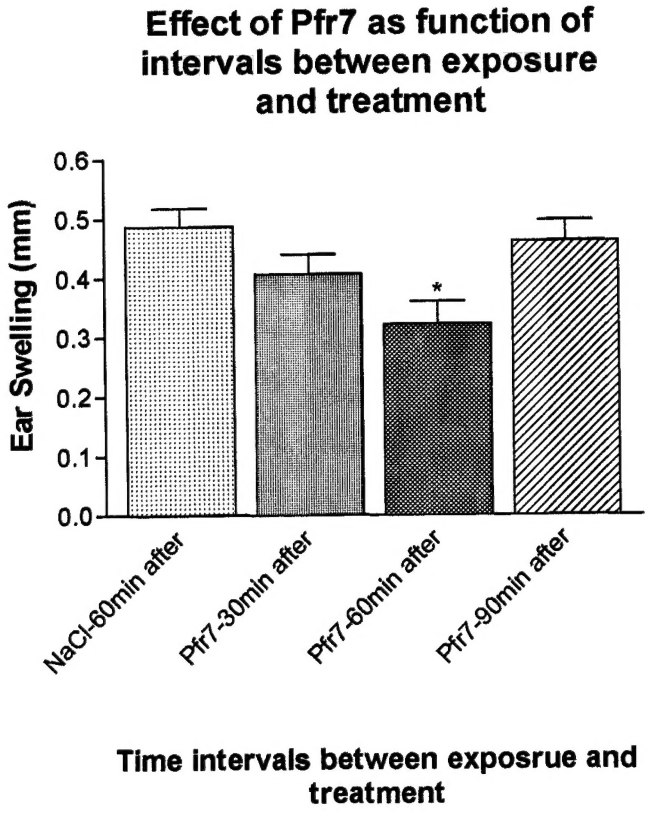
Peptide was injected sc 30 min after exposure.
*p<0.02

Fig. 13



Peptides were injected sc (into the ear base) 30 min after exposrue. *p<0.03

Fig. 14



The peptide was injected sc in to the ear base at dose of 50μg per site of injection. Measrement was carried out 4 days after treatment. *p<0.005